

In Vitro Selection of DNA Elements Highly Responsive to the Human T-Cell Lymphotropic Virus Type I Transcriptional Activator, Tax

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The human T-cell lymphotropic virus type I (HTLV-I) transactivator, Tax, the ubiquitous transcriptional factor cyclic AMP (cAMP) response element-binding protein (CREB protein), and the 21-bp repeats in the HTLV-I transcriptional enhancer form a ternary nucleoprotein complex (L. J. Zhao and C. Z. Giam, Proc. Natl. Acad. Sci. USA 89:7070-7074, 1992). Using an antibody directed against the COOH-terminal region of Tax along with purified Tax and CREB proteins, we selected DNA elements bound specifically by the Tax-CREB complex in vitro. Two distinct but related groups of sequences containing the cAMP response element (CRE) flanked by long runs of G and C residues in the 5' and 3' regions, respectively, were preferentially recognized by Tax-CREB. In contrast, CREB alone binds only to CRE motifs (GNTGACG[T/C]) without neighboring G- or C-rich sequences. The Tax-CREB-selected sequences bear a striking resemblance to the 5' or 3' two-thirds of the HTLV-I 21-bp repeats and are highly inducible by Tax. Gel electrophoretic mobility shift assays, DNA transfection, and DNase I footprinting analyses indicated that the G- and C-rich sequences flanking the CRE motif are crucial for Tax-CREB-DNA ternary complex assembly and Tax transactivation but are not in direct contact with the Tax-CREB complex. These data show that Tax recruits CREB to form a multiprotein complex that specifically recognizes the viral 21-bp repeats. The expanded DNA binding specificity of Tax-CREB and the obligatory role the ternary Tax-CREB-DNA complex plays in transactivation reveal a novel mechanism for regulating the transcriptional activity of leucine zipper proteins like CREB.

Human T-cell lymphotropic virus type I (HTLV-I) is the prototype of a group of retroviruses (including HTLV-I, HTLV-II, simian T-cell leukemia virus, and bovine leukemia virus [BLV]) that produce regulatory proteins to modulate viral mRNA synthesis and utilization (6). The 40-kDa nuclear protein, Tax, encoded by the 3' region of the HTLV-I genome is a transcriptional activator (6). Tax does not bind DNA directly (13, 21). Like many other viral transactivators such as herpes simplex virus VP16 and adenovirus E1a (for a review, see reference 20), Tax forms multiprotein complexes with host cell transcription factors to gain control of the cellular mRNA synthetic machinery for viral replication (1, 18, 27-29). The ability of Tax to alter gene expression in HTLV-I-infected cells appears to be causally linked to HTLV-I pathogenesis, which is manifested clinically as adult T-cell leukemia (5, 16, 24) and tropical spastic paraparesis/HTLV-I-associated myelopathy (2, 12, 17, 22).

Tax activates viral transcription from three 21-bp repeats in the U3 region of the HTLV-I long terminal repeat (3, 23, 25, 26). Mutational analyses indicated that two copies of the repeat and the CRE (cyclic AMP [cAMP]-responsive-element)-like sequences, TGACG, in the repeats are crucial for transactivation by Tax (3, 13, 23, 25, 26). Although their functions have not been well defined, the DNA sequences surrounding the CRE motif also play an important role in

Tax action (10, 19). This is best shown by the inability of HTLV-I Tax to transactivate three CRE-containing 21-bp repeats in the transcriptional regulatory region of a related retrovirus, BLV (7, 8). The BLV repeats are distinct from their HTLV counterparts in that their CREs adjoin unique 5' and 3' flanking sequences and that they are activated only by the 36-kDa BLV transactivator, BLV Tax (7). We previously showed that via a direct protein-protein interaction, HTLV-I Tax formed multiprotein complexes with the ubiquitous transcription factor CRE binding protein (CREB protein), and with the CREB-ATF-1 (activating transcription factor 1) heterodimer (29). The interaction between Tax and CREB is highly specific. Despite the high degree of amino acid sequence homology between CREB and ATF-1, Tax interacts efficiently with the CREB homodimer or the CREB-ATF-1 heterodimer but not with the ATF-1 homodimer (29). The Tax-CREB complexes assembled specifically on the HTLV-I 21-bp repeats to produce protein-DNA complexes at levels much higher than when CREB or CREB-ATF-1 alone was used (29). Here we describe the in vitro selection of highly Tax-inducible elements. We demonstrate that the Tax-CREB complex exhibits much greater DNA recognition specificity than CREB. The consensus Tax-CREB-selected binding site, GGGGG(T/G)TGACG(T/C)(A/C)TA(T/C)CCC CC, is highly homologous to the HTLV-I 21-bp repeats. Further, the G- and/or C-rich sequences adjacent to the CRE motif are shown to be crucial for the formation of a stable Tax-CREB-DNA ternary complex and Tax transactivation. These results indicate that HTLV-I employs the viral transactivator Tax to convert the ubiquitous cellular transcription

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factor CREB to a form that can specifically assemble on the viral regulatory element to facilitate viral replication.

MATERIALS AND METHODS

In vitro binding site selection. Tax and CREB were purified from bacterial expression systems as previously described (29). A 47-mer oligonucleotide, GCCG CTCGAG AGAT CT(N15)CCATGG CATATG GCCG, containing 15 random bases flanked by 5' *Xho*I and *Bgl*II and 3' *Nco*I and *Nde*I restriction site sequences was made double stranded by primer extension with a 16-mer complementary to its 3' end and the bacteriophage T4 DNA polymerase. The binding reaction was carried out for 30 min at 37°C with 2 µg of CREB, 3 µg of Tax, 1 µg of 47-bp fragments, and 5 µg of poly(dI-dC) in 60 µl of binding buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.9], 20 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 mg of bovine serum albumin per ml, 10% glycerol, 1% (Nonidet P-40). An antibody (TaxC Ab) generated against the peptide (TaxC peptide) containing the COOH-terminal 33 amino acid residues of Tax (FNEKEADENDHEPOIS PGGLEPPSEKHFRETEV) was used to capture the ternary Tax-CREB-DNA complexes. TaxC Ab (1 µl) was bound to protein A-Sepharose beads (30 µl) in 100 µl of phosphate-buffered saline (PBS) containing 6 µg of poly(dI-dC) and 0.1% Nonidet P-40 for 2 h at room temperature. The TaxC Ab-charged beads (15 µl) were washed with 1 ml of PBS three times, pelleted, and added to the binding reaction mixture, which was then incubated for 1 h at 4°C. The beads were then centrifuged and washed five times with 500 µl of binding buffer and once with 50 µl of 0.6 M NaCl in Tris-EDTA (10 mM Tris [pH 7.5], 1 mM EDTA) buffer. The bound DNA was eluted in 20 µl of 3 M NaCl in Tris-EDTA buffer, diluted 100-fold, and amplified by 15 cycles of PCR with a pair of 16-mers prepared according to the invariant sequences on both ends of the 47-mers. Five cycles of selection were performed. Selection of CREB binding sites was performed similarly to that for Tax-CREB with the same pool of random oligonucleotides. The CREB-bound DNA was captured on nitrocellulose filter paper under suction. The filter was rinsed four times with 100 µl of binding buffer, and the bound DNA was eluted with 150 µl of 3 M NaCl in Tris-EDTA buffer. The eluate was diluted 100-fold and amplified by 15 cycles of PCR. Again, a total of five selection-amplification cycles was performed. The selected DNA fragments were cloned via cleavage at the *Nco*I (CCATGG) and *Bgl*II (AGATCT) sites and sequenced by the dideoxy chain termination method.

Construction of CAT plasmids and CAT assay. CREs with distinct flanking sequences were duplicated by PCR and cloned via *Xho*I and *Bgl*II sites upstream of the chloramphenicol acetyltransferase (CAT) indicator gene. The G- and C-rich regions in the CRE flanked by 6 C residues (C6) and the one flanked by 10 G residues (G10) are oriented proximal to the TATA box. Transfections and CAT assays were carried out with Jurkat T cells as described previously (28) by the DEAE dextran method with 3 µg of the test constructs and 2 µg of a Tax expression plasmid, pHTLV-I Tax.

Gel electrophoretic mobility shift assay. Gel electrophoretic mobility shift assays were carried out with purified Tax and CREB proteins and various p32-labeled DNA fragments containing the CRE motifs in Tris-glycine buffer at 4°C as described previously (28, 29).

DNase I protection. The HTLV-I 21(1) repeat flanked on the 5' and 3' ends by *Bgl*II, *Nco*I, and *Nde*I restriction sites

was cloned on the pUC18 plasmid vector into the *Sma*I site and used in the footprinting analyses. The *Eco*RI-*Nco*I DNA fragment containing the 21(1) sequence, labeled at the 3' end of the antisense strand, was incubated for 20 min with various amounts of CREB and 100 ng of Tax. DNase I (2 µl from a 1-U/µl stock solution in 0.25 mM CaCl₂; Pharmacia) was added to the binding reaction mixtures for 5 min. The reactions were stopped by adding 15 µl of a buffer containing 0.75% sodium dodecyl sulfate, 100 mM Tris (pH 7.8), 15 mM EDTA, 0.6 M sodium acetate, and 25 µg of yeast tRNA per ml. The DNA was phenol extracted, precipitated with 2 volumes of ethanol, and analyzed on a 10% polyacrylamide sequencing gel.

Methylation interference. The *Eco*RI-*Nco*I probe labeled at either the sense or the antisense strand was partially methylated with dimethyl sulfate, purified, and incubated with CREB or with Tax and CREB. The bound and unbound probes were resolved by gel electrophoretic mobility shift assay. They were then cut out of the gel, purified by electroelution, treated with piperidine by Maxam and Gilbert procedures, and analyzed on a 10% polyacrylamide sequencing gel.

RESULTS

In vitro selection of DNA sequences preferentially bound by the Tax-CREB complex. We reported earlier that an antibody, TaxC Ab, raised against a peptide containing 33 amino acid residues in the COOH terminus of Tax specifically precipitated the Tax-CREB complex (29). This provides a means to select for DNA elements that interact specifically with Tax-CREB. For this purpose, a 47-mer oligonucleotide, GCCGCTCGAGAGATCT(N15)CCATGGCATATGGCCG, containing 15 random bases flanked by 5' *Xho*I and *Bgl*II, and 3' *Nco*I and *Nde*I restriction site sequences was prepared, made double stranded by primer extension, and incubated with purified Tax and CREB. The ternary complexes were then precipitated with the TaxC Ab adsorbed to protein A-Sepharose beads. The bound DNA was eluted and amplified by PCR, using a pair of 16-mers as primers. Five rounds of binding site selection were carried out. The selected DNA fragments were digested with *Bgl*II and *Nco*I restriction enzymes and inserted into a pUC18 derivative. A total of 34 plasmid clones was sequenced by the dideoxy chain termination method, and the results are listed in Fig. 1A. The Tax-CREB binding sequences fall into two classes designated groups C and G. Each sequence in group C is composed of a CRE-like motif (TGACGC) followed by three or four A/T bases and a long stretch of C residues in the 3' end. Group G sequences typically contain long runs of G residues at their 5' ends, followed immediately by a T or G residue and the CRE motif. The sequence marked with an asterisk in group C exhibits characteristics of both groups. The consensus sequence for each group was deduced. The group G consensus resembles the 5' two-thirds of the HTLV-I 21-bp repeats, while the group C consensus resembles the 3' two-thirds. These two sequences can be joined through the CRE overlap to give an element (a joined consensus [JC]) containing an 11-bp core, (T/G)TGACG(T/C)(A/C)TA(T/C), flanked by five G and five C residues on the 5' and 3' sides, respectively. All three HTLV-I 21-bp repeats, and the promoter-proximal repeat 21(1) in particular, display a high degree of sequence homology to the JC. To investigate whether the preference for CRE motifs with G- and/or C-rich flanking sequences is a unique attribute of the Tax-CREB complex, we carried out binding site selection

A**Tax/CREB Selected Binding Sites**

| | |
|--|-------------------------|
| <u>AGATCTATGACGCATATCCCCCATGG</u> | (10) |
| AGATCTATGACGCATACCCCCCATGG | (2) |
| AGATCTAGTGACGCACCCCCCATGG | (1) |
| AGATCTTATGACGCATATCCCCCATGG | (1) |
| CCATGGGGTGACGCATACCCATAGATCT | (1)* |
| TGACGCATA^TCCCCC_C | CONSENSUS C |
| GGGGG^TTGACG^{TA}_{CC} | CONSENSUS G |
| CCATGGGGGGGGGTTGACGTAGATCT | (2) |
| AGATCTCCGGGGCGGTTACGTCCATGG | (2) |
| AGATCTGGGGGGTGACGGAGCCCATGG | (1) |
| CCATGGATGGGGGTTGACGTAGATCT | (1) |
| CCATGGGCAGGGGTTGACGTAGATCT | (1) |
| AGATCTATTGGGTGGTTGACGCCATGG | (1) |
| CCATGGGGGTTACGCATATCGAGATCT | (1) |
| AGATCTCCGAGCGGGTTGACGCCATGG | (1) |
| AGATCTAGGAAGGGGTTGACGCCATGG | (1) |
| AGATCTGTGGGTGACGTACGCCATGG | (1) |
| CCATGGCGGTGACGGTACGTAGATCT | (1) |
| AGATCTGGGATTACGTATCCATGG | (1) |
| AGATCTAGGGTTGACGTACGCCATGG | (1) |
| AGATCTCGGGACTGACGCATGCCATGG | (1) |
| AGATCTATGGGTGACGCATGCCATGG | (1) |
| CCATGGCTAGACGGTTGACGCAGATCT | (1) |
| AGATCTCCGGCTGACGACAATCCATGG | (1) |
| GGGGG^TTGACG^TTA^TCCCCC_C | JOINED CONSENSUS |
| GGGCGTTGACGACAACCCCTC | HTLV 21(1) |
| AGGCCCTGACGTCTCCCCCTG | HTLV 21(2) |
| AGGCTCTGACGTCTCCCCCG | HTLV 21(3) |

B**CREB Selected Binding Sites**

| | |
|--|-----------------------|
| <u>AGATCTTGATGACGCAATACGCCATG</u> | (1) |
| AGATCTCGGGGTATGTTGACGCCATGG | (1) |
| AGATCTTCCAGCGGGTGACGCCATGG | (1) |
| AGATCTGTTCGGATGACGTCCCATGG | (1) |
| CCATGGGATGACGGTCCCCGAGATCT | (1) |
| AGATCTGGGTGACGACAATCCCATGG | (1) |
| CCATGGACAATTGGATGACGTAGATCT | (1) |
| AGATCTTGGCGGGTGACGTTCTCCATGG | (1) |
| CCATGGGATGCCCGCTTACGTAGATCT | (1) |
| AGATCTGATGACGCGATGCCCCCATGG | (1) |
| AGATCTGGCGGGTGACGTGTGCCATGG | (1) |
| AGATCTTGACGTAGTTGAGTTCCATGG | (1) |
| AGATCTCGGGGACGTGACGTACCATGG | (1) |
| AGATCTTACGTAGTACGTGCGCCATGG | (1) |
| AGATCTTGGATGACGTGCGGATCCATGG | (1) |
| AGATCTTCGTGACGTTCTTGGCATGG | (1) |
| GNTGACG^T_C | CREB CONSENSUS |

FIG. 1. Sequence comparison between binding sites selected with Tax-CREB (A) and CREB (B). Two groups of binding sites were recognized by the Tax-CREB complex. Sequences within each group were arranged in descending order according to the frequency of occurrence (indicated in parentheses) and the number of C or G residues that appeared in the vicinity of the CRE motifs. The consensus sequences from groups C and G (each ended at the borders of the *Bgl*III and *Nco*I sites, respectively) were joined through the CRE overlap to yield the JC element. The sequences of all three HTLV-I 21-bp repeats are listed for comparison. The 21(1) repeat is situated proximal to the HTLV-I TATA box. The sequences underlined and the JC were chosen for further characterization. CREB-selected sequences were arranged in descending order according to their homology to the CREB consensus.

with CREB protein alone. The selection-PCR amplification cycles and DNA sequencing are detailed in Materials and Methods. After examination of 16 CREB-selected sequences (Fig. 1B), a consensus CREB binding site, GNTGACG(T/C), without adjoining G- or C-rich sequences was deduced. This result contrasts with that obtained with Tax-CREB and

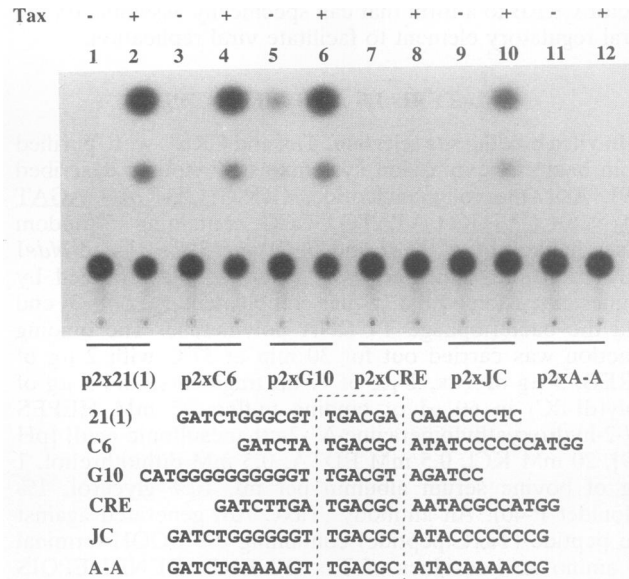


FIG. 2. Response of in vitro-selected binding sites and their derivatives to Tax transactivation. The DNA sequences of the HTLV 21-bp repeat [21(1)], C6, G10, a CREB-selected binding site (CRE), the JC, and the A-A mutant are listed, with the CRE motifs boxed. All constructs tested contain two tandem copies of the respective DNA elements upstream of the HTLV-I minimal promoter (nucleotides -67 to +268) and the CAT reporter gene. The HTLV-I minimal promoter-CAT construct showed little basal activity and was not responsive to Tax (data not shown). The percent conversion of [¹⁴C]chloramphenicol to the acetylated forms for lanes 1 to 12 are 0.1, 59.6, 0.1, 52.9, 4.6, 52.5, 0.3, 1.2, 0.15, 18.1, 0.3, and 0.3, respectively.

indicates that Tax-CREB recognizes specifically the subset of CRE motifs with G- and/or C-rich flanking sequences.

Tax-CREB binding sites are highly transactivated by Tax and require both the CRE motif and the adjacent G- and/or C-rich sequences for Tax activation. To determine whether the selected binding sites can be transactivated by Tax, we constructed plasmids containing duplicate CRE motifs upstream of the HTLV-I minimal promoter and the indicator CAT gene. These plasmids were cotransfected with a Tax expression construct, pHTLV-I-tax (28), into Jurkat cells, and the levels of transactivation by Tax were determined. The CRE flanked by 6 C residues (C6) and the one flanked by 10 G residues (G10), both underlined in Fig. 1A, were chosen for analysis because they represent the most frequently occurring sequences in their respective groups. As shown in Fig. 2, similar to the HTLV-I 21-bp repeats (lanes 1 and 2), both p2xC6 and p2xG10 were highly transactivated by Tax (compare lanes 3 and 4, and lanes 5 and 6). In contrast, a sequence containing the CRE consensus and picked from the CREB-selected binding sequences (underlined in Fig. 1B) was unresponsive to Tax (Fig. 2, lanes 7 and 8). We next used the JC as a representative of all Tax-CREB-selected binding sites and examined its Tax inducibility. As expected, the JC is activated by Tax (Fig. 2, lanes 9 and 10). When the flanking G and C bases in the JC were replaced with A residues, the resultant element, A-A, lost Tax inducibility completely (compare lanes 11 and 12 in Fig. 2).

The assembly of the Tax-CREB-DNA ternary complex correlates with Tax transactivation and requires DNA elements containing a CRE motif and flanking G and/or C-rich

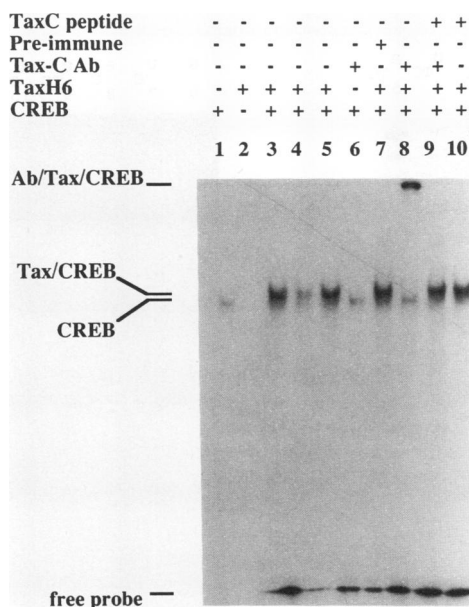


FIG. 3. In vitro assembly of the Tax-CREB-JC DNA ternary complex. A 32-bp *Bgl*II-*Nco*I DNA fragment containing the JC sequence was 3' end labeled and used as a probe. Equal amounts of probe DNA were used in all lanes. In lanes 1 through 3, CREB alone (20 ng), Tax alone (100 ng), and both Tax and CREB were added, respectively. The free probe for lanes 1 and 2 migrated out of the gel. In lanes 4 and 5, Tax and CREB were added together with 5 ng of competitor DNA: a *Bgl*II-*Nco*I fragment containing the 21(1) repeat (lane 4) (see Fig. 2 for the sequence) and a 21(1) mutant with a mutated CRE motif, GGACG (lane 5). In lane 6, CREB and TaxC Ab were added. Lanes 7 through 10 contained Tax and CREB but with preimmune serum (0.5 μ l), TaxC Ab (0.5 μ l), TaxC antibody plus blocking TaxC peptide (0.2 μ g), and TaxC peptide alone, respectively.

sequences. To investigate further the effects of the flanking regions on the assembly of the Tax-CREB-DNA complex, we carried out gel electrophoretic mobility shift assays with the JC DNA as a probe. CREB bound the JC DNA probe and produced a nucleoprotein complex (Fig. 3, lane 1), while Tax did not (lane 2). The addition of Tax and CREB together yielded at an increased level a ternary complex (marked Tax/CREB) with a lower mobility than that of CREB alone (compare lanes 1 and 3 in Fig. 3). As previously reported (28, 29), complex formation could be inhibited by the wild-type 21(1) repeat (Fig. 3, lane 4) but not by a repeat containing a CRE mutation (lane 5). Finally, the presence of Tax in the complexes was demonstrated by mobility supershift with the TaxC Ab (compare lanes 6, 7, and 8 in Fig. 3). As expected, the TaxC Ab did not affect CREB binding (Fig. 3, lane 6), and the preimmune serum had no effects on the ternary complex (lane 7). The TaxC peptide blocked the TaxC Ab supershift effectively (compare lanes 8, 9, and 10 in Fig. 3). Six DNA probes, including the HTLV-I 21-bp repeat [21(1)], C6, G10, a CREB-selected sequence (CRE), the JC, and the A substitution mutant (A-A), were analyzed (see Fig. 2 for sequences). As shown in Fig. 4, all DNA probes formed complexes with CREB and migrated to positions as marked (lanes 1, 4, 7, 10, 13, and 16). For reasons which are unclear at present, the CREB-CRE complex migrated more slowly in the gel (Fig. 4, lanes 10 to 12). In agreement with earlier results (28, 29), Tax also increased the levels of nucleopro-

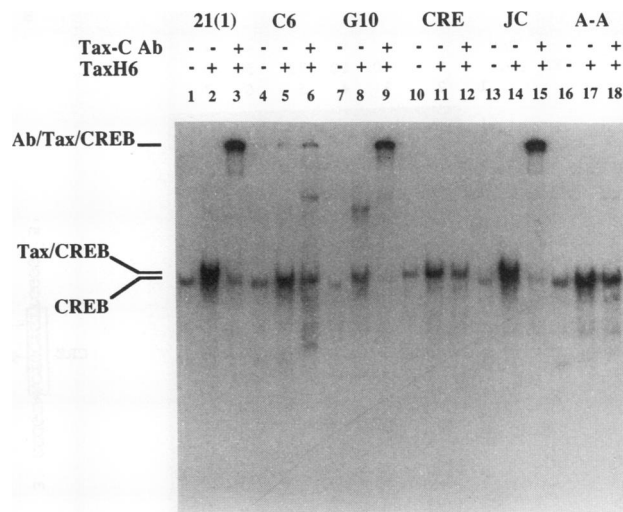


FIG. 4. Formation of the Tax-CREB-DNA ternary complex requires the G- and C-rich flanking sequences. DNA binding reactions and gel electrophoretic mobility shift assays were carried out for six 32-bp *Bgl*II-*Nco*I DNA fragments each containing either the HTLV-I 21(1), C6, G10, CRE, JC, or A-A sequences (sequences listed in Fig. 2) (three lanes in each set). All reaction mixtures contained CREB. Lanes 1, 4, 7, 10, 13, and 16 contained CREB alone; lanes 2, 5, 8, 11, 14, and 17 contained Tax and CREB; and lanes 3, 6, 9, 12, 15, and 18 contained Tax, CREB, and TaxC Ab.

tein complexes for all probes (compare within each set of three). As described previously (28), this activity was lost after pretreatment at 70°C for 5 min, and this loss could not be explained by trivial possibilities such as buffer effects from the Tax preparation. More-slowly-migrating ternary complexes (marked Tax/CREB) were detected for the Tax-inducible HTLV-I 21(1), G10, and JC DNA probes and, to a lesser extent, the C6 probe (Fig. 4, lanes 2, 8, 14, and 5, respectively). The nonresponsive CRE and A-A mutant probes did not yield detectable levels of ternary complexes (Fig. 4, lanes 11 and 17). The presence of Tax in the complexes was further confirmed by their mobility supershift by the TaxC Ab (marked Ab/Tax/CREB) (Fig. 4, lanes 3, 6, 9, and 14). Thus, qualitatively, the assembly of a Tax-CREB-DNA ternary complex correlates with Tax trans-activation and requires the CRE and flanking G- and/or C-rich regions. Further, although Tax enhanced CREB binding to the CRE and the A-A mutant probes, the failure of these elements to be activated by Tax coincided with their inability to assemble ternary complexes with Tax-CREB in the gel shift assays.

The Tax-CREB complex does not directly contact the G- and C-rich sequences flanking the CRE motifs in Tax-responsive elements. Using DNase I protection and methylation interference techniques, we tested whether the Tax-CREB multiprotein complex directly contacts the G- and C-rich flanking sequences in Tax-responsive elements. The 21-bp repeat, 21(1), of HTLV-I was uniquely 3' end labeled and used as a probe. As shown in Fig. 5A, the CRE region in the probe DNA was protected from DNase I digestion by CREB (compare lanes 3 and 5). The pattern of protection, however, was not significantly altered by the addition of Tax (Fig. 5A, lanes 5 and 6). For reasons which are unclear at present, Tax renders the TATA-proximal region in the probe more DNase I sensitive (compare the upper parts in Fig. 5A, lanes 3 and 4, and lanes 5 and 6). In agreement with results from the

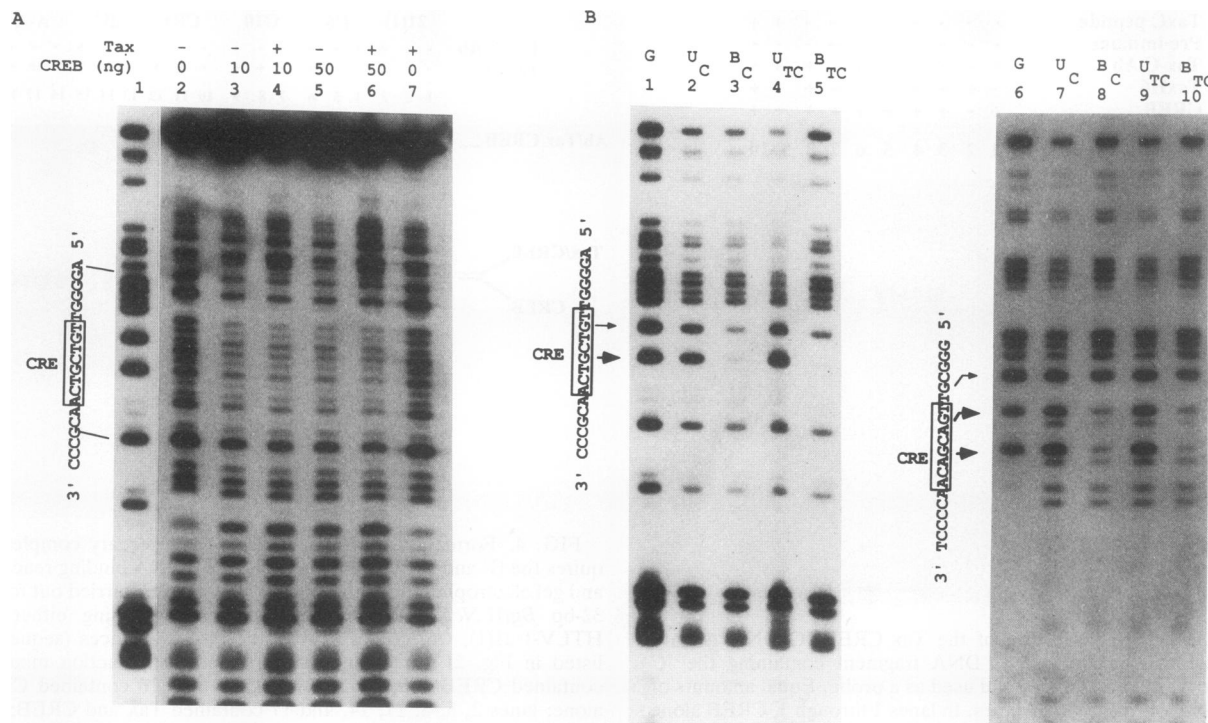


FIG. 5. CREB and Tax-CREB produce similar footprints on 21(1) DNA. (A) DNase I protection. Lanes 2 and 7, 3 and 4, and 5 and 6 contained 0, 10, and 50 ng of CREB, respectively. Tax (100 ng) was added in lanes 4, 6, and 7. (B) Methylation interference. The *EcoRI-NcoI* probe was labeled at either the sense (lanes 6 through 10) or the antisense (lanes 1 through 5) strand. Lanes: 1 and 6, methylated DNA directly cleaved with piperidine (G); 2 and 7, unbound DNA (U_C); 3 and 8, bound DNA (B_C) when CREB alone was used; 4 and 9, unbound DNA (U_{TC}); 5 and 10, bound DNA (B_{TC}) when both Tax and CREB were used. The 21(1) sequence is shown on the side for reference. Large arrows mark the residues critical for protein binding. Small arrows denote residues whose methylation resulted in reduced binding.

binding site selection, methylation interference analysis indicated that both CREB binding and Tax-CREB binding to 21(1) required all three G-C base pairs in the TGACG motif (large arrows in Fig. 5B) (for CREB, compare lanes 2 and 3 [antisense strand] and 7 and 8 [sense strand]; for Tax-CREB, compare lanes 4 and 5 [antisense strand] and 9 and 10 [sense strand]). The two G-C base pairs marked with small arrows also interacted with CREB and Tax-CREB (Fig. 5B, lanes 7 through 10) (compare the relative intensities of these residues and those of the three G's upstream in lanes 7 and 8, and lanes 9 and 10). This is again consistent with the binding site selection showing that both CREB and Tax-CREB prefer G-C pairs in these two positions. Guanine methylation of the G- and C-rich sequences surrounding the GTTGAC GACAA core, however, did not result in any overt effects on CREB or Tax-CREB binding to 21(1). As shown in Fig. 5B, the methylation interference patterns produced with CREB and Tax-CREB are virtually identical (compare lanes 3 and 5, and lanes 8 and 10). These analyses suggest that CREB most likely constitutes the primary DNA binding component of the Tax-CREB complex.

DISCUSSION

Like other members of the bZIP family of DNA-binding proteins, CREB interacts with DNA as a dimer (4, 14). Earlier results show that the molecular size of Tax (40 kDa) is similar to that of ovalbumin (43 kDa), indicating that Tax is monomeric (13). Because Tax lacks any bZIP motif in its protein sequence, we think it unlikely that Tax would dimerize with CREB to form a heterodimer like Jun-Fos.

This was borne out by earlier results showing that Tax formed a distinct complex with the CREB-ATF-1 (15) heterodimer but not with the ATF-1 homodimer (29). We speculate that the selected binding sites and the full 21-bp repeat interact with the Tax-CREB complexes containing both a CREB homodimer and either one molecule or two molecules of Tax, respectively. The selection of the 5' or 3' two-thirds of a full 21-bp repeat is apparently due to the constraint imposed by having only 15 random bases in the 47-mer DNA used in the selection. The lack of a perfect dyad symmetry in the CREB-selected CRE sites implies that the interactions of individual subunits in the CREB homodimer with DNA are not identical. This is also reflected in the Tax-CREB-selected group C and G sequences, the JC sequence, and the 21(1) repeat. Tax by itself does not interact with DNA. If a DNA binding domain existed in Tax, it could be unmasked only after interacting with CREB. The absence of detectable base-specific contact between the Tax-CREB complex and the flanking G and C sequences is intriguing. The methylation interference experiment ruled out Tax-CREB interaction with the G at N-7 in the major groove. However, the possibility that Tax-CREB contacts the G residues at the N-3 position in the minor groove remains. Alternatively, the long runs of G and C residues may affect DNA conformation in the vicinity of or within the CRE motif so that the structurally altered CRE could be recognized efficiently only by Tax-CREB. A conformational role for the G- and C-rich regions would be consistent with a previous analysis of the 21-bp repeat by saturation mutagenesis which showed that single base substitutions in these sequences did

not overtly affect Tax transactivation (13). We and others have shown that Tax and CREB form a complex in the absence of DNA (27, 29). How can this result be reconciled with the inability of the mutant A-A and the CREB-selected site to assemble ternary complexes with Tax-CREB and with the enhancement of CREB binding to these two sequences by Tax? It is possible that, in general, Tax-CREB may bind CRE more avidly. However, only binding to the CREs with suitable flanking sequences stabilizes further Tax-CREB interaction and leads to stable ternary complex formation as detected by the TaxC Ab supershift, while binding to CREs with undesirable flanking sequences causes Tax dissociation. Qualitatively, the formation of the Tax-CREB-DNA ternary complex correlated with Tax transactivation, suggesting that the physical presence of Tax in the complex is important for transactivation. This is in agreement with reports showing that Tax contains a transactivation domain, demonstrated with the Gal4-Tax fusion protein (9, 11). The level of ternary complex formation with the C6 probe, as determined by the gel shift assay, however, did not reflect this sequence's frequency of occurrence in the Tax-CREB-selected sites or its level of transactivation by Tax. The same effect was also observed for DNA probes containing the 21(2) and 21(3) repeats (data not shown). We think the conditions under which the gel shift assays were performed may cause partial dissociation of complexes that were otherwise stable under more physiological conditions. In summary, our study shows that the interaction between Tax and CREB results in the formation of a multiprotein complex with far greater DNA recognition specificity than that of CREB alone. The similarity between the Tax-CREB-selected binding sites and the HTLV-I 21-bp repeats lends additional support to the notion that CREB is a major protein target of Tax. These results are relevant for understanding the molecular mechanism of HTLV-I pathogenesis. Furthermore, the increased DNA binding specificity of Tax-CREB also implicates a novel mechanism for modulating the specificity and transcriptional activity of the large family of CREB/ATF proteins.

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